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COMPOUNDS COMPRISING PSEUDO-AMINO ACIDS

This invention relates to compounds, and in particular to
polypeptides, their use and methods for their production.
These polypeptides are produced from "pseudo-cysteine" amino
acids that facilitate the assembly of polypeptides by native
chemical ligation.

Background

It is known that it is possible to ligate two peptidic or non-
peptidic compounds together by a process known as native
chemical ligation. The basic requirements for this coupling
are the presence on participant **A** of a thioester moiety such
as a benzyl or ethyl thioester, and the presence on
participant **B** of an N-terminal cysteine. The coupling
reaction commences when the thio-nucleophile initially attacks
the thioester in a reversible fashion causing the thioalkyl
group to leave. The thioester so formed then undergoes an
intramolecular S→N acyl shift to irreversibly produce the
ligated amide product **A-B** with a native cysteine at the
junction (Figure 1).

This process can be repeated if it is possible to unmask a
further N-terminal cysteine at the N-terminal of **A**, in the
product **A-B**. This can be achieved by enzymatic means or by
classical protecting group chemistry. A further native

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chemical ligation coupling with a thioester-containing moiety

E would therefore yield the product E-A-B. These methods have

been used to generate a variety of proteins.

It is also known that cyclic peptides/proteins can be produced

by incorporating both the thioester and amino terminal

cysteine (N-terminal cysteine) moieties within the same

molecule.

Further, it is possible to perform the ligation described

above without removing all of the reaction participants from a

solid phase.

A method for incorporating a thioester moiety into a

participant where one does not exist is also known. This

method relies on the reaction of a primary amine with

thiolane-2,5-dione and subsequent alkylation of the thioacid

formed with an appropriate alkyl bromide. This method

requires a single primary amine to be present otherwise

multiple thioesters may be coupled together.

Amino-acid monomer molecules are available with a protected

functionality grafted onto another amino acid sidechain. For

example, N-α-Fmoc-N-β—(N-t-Boc-amino-oxyacetyl)-L-

diaminopropionic acid (Novabiochem - San Diego, CA)

incorporates a protected oxyamine onto an amino acid sidechain

that is capable, after deprotection, of undergoing

chemoselective ligation to carbonyl containing moieties.
However, with reagents currently available it is not possible
to incorporate a pseudo C-terminal cysteine into a
polypeptide. A C-terminal cysteine is desirable to allow the
linking of a molecule to a thioester (*vide supra*) without
using an N-terminal cysteine, if for example, the N-terminus
is required unmodified for biological activity.

Further, there is no method to enable the ligation of a C-
terminal or other thioester with a pseudo C-terminal cysteine.

We have now produced reagents that enable the incorporation
onto an amino acid sidechain at the C-terminal end of the
peptide, a surrogate 1-amino-2-thiol that would be capable of
performing a native chemical ligation reaction.

Further, we have disclosed a method that can be used to
introduce a 1-amino-2-thiol group into the side chain of a
lysine to enable the conjugate of a polypeptide containing
such a residue to be ligated to a target compound using native
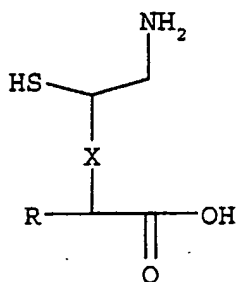chemical ligation methods.

Detail of the Invention

The invention provides a compound comprising a polypeptide,
the polypeptide having at a C-terminal end a pseudo amino

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acid, the pseudo amino acid having a side chain containing a

1-amino-2-thiol moiety.

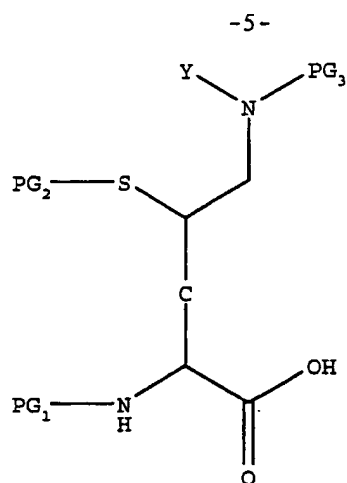Further it provides a compound having a polypeptide of the

structure:

$$
\begin{array}{c}
NH_2 \\
HS \underset{\displaystyle |}{\overset{\displaystyle |}{\diagdown}} \\
X \\
R \underset{\displaystyle \underset{O}{\parallel}}{\overset{\displaystyle |}{\rule{0pt}{0pt}}} OH
\end{array}
$$

wherein:

R is a polypeptide chain;

X is a linker.

The linker may comprise, for example, methylene units i.e. CH_2

units or may include residues covalently linked by amide,

ester, ether, thioether bonds or aryl groups, for example.

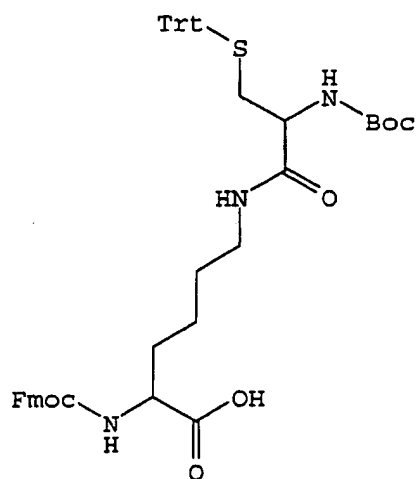Further, the linker may be (CH2)n, where n is 0 to 6,

preferably 4.

The invention also provides a protected pseudo amino acid

comprising the structure

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$$
\begin{array}{c}
Y \diagdown \underset{|}{N} \diagup PG_3 \\
PG_2 - S \diagup \underset{|}{C} \\
\underset{|}{C} \\
PG_1 - \underset{H}{N} \quad C \diagup OH \\
\parallel \\
O
\end{array}
$$

where the Y is optionally H or other suitable residue and PG1, PG2 and PG3 are different protecting groups.

The protecting groups may be FMOC, BOC or Trt, for example although other will be known to those skilled in the art.

One suitable protected amino acids is

$$
\begin{array}{c}
Trt \diagdown S \\
\underset{N}{H} \diagdown Boc \\
HN \diagup \underset{\parallel}{C} \diagdown O \\
Fmoc \diagdown \underset{H}{N} \quad \diagup OH \\
\parallel \\
O
\end{array}
$$

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which may be produced as described in Example 2 and
illustrated in Figure 2.

WO 98/02454 relates to the derivatisation of soluble
complement regulators by disulfide exchange reaction with
pyridyl disulfide activated myristoyl/electrostatic switch
peptides (MSWPs). These derivatives demonstrate improved
complement inhibitory activity owing to their membrane
localisation by the MSWP. The myristoyl group was designed to
be minimally membrane insertive, while the lysine rich
peptidic portion of MSWP-1 (Seq. ID No 1) was designed to
interact with the negatively charged phospholipid headgroups
present on the lipid bilayer. The concept is exemplified in
WO 98/02454 by SCR1-3 of LHR-A of CR1, altered by a single C-
terminal cysteine residue. After treatment with an
appropriate reducing agent to ensure the presence of a free
thiol on the terminal cysteine sidechain, the protein is
reacted with MSWP-1 (N-(Myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-
Ser-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-(S-2-Thiopyridyl)Cys-
NH$_2$) (Seq ID No: 1) to yield a membrane binding derivative of
SCR1-3 (Figure 4). The resultant conjugate protein showed
anti-haemolytic activity orders of magnitude greater than that
displayed by the underivatised protein alone.

This chemistry relies on the existence within the protein
participant of the reaction a single free thiol resulting in
derivatisation at a single point only. In other circumstances

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it is desirable to link the class of peptide described to

proteinaceous or non-proteinaceous molecules bearing a

thioester. In the current design of the peptide, however, an

N-terminal myristoyl function is required, thus disallowing

the incorporation of an N-terminal cysteine to participate in

the ligation reaction.

In order to achieve this, we describe an amino acid monomer

capable of participating in standard Fmoc synthesis that would

yield, on final deprotection a 1-amino-2-thiol at the c-

terminus (Figure 2 and Example 2). When incorporated into a

polypeptide containing myristoyl switch type peptide (APT2278

– Seq ID No: 2), it was indeed found to be capable of

undergoing native chemical ligation to three widely differing

thioester-containing molecules, a vitamin, a polypeptide, and

an Impact™ expressed protein thioester.

Also disclosed is the incorporation of such a protected

sidechain 1-amino-2-thiol into a peptide by the use of

orthogonal protecting groups (Example 3).

Where the thioester-containing moiety is a protein, one method

of formation of the ligated product of the invention is

provided by the IMPACT system (New England Biolabs).

Conjugates are afforded by the native chemical ligation of

peptides containing an N-terminal cysteine, to proteins

expressed in frame with a controllable intervening peptide

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sequence (CIVPS) and an affinity purification handle. The desired protein is cleaved from its affinity purification matrix by elution with a free thiol such as MESNA to yield a C-terminal thioester. The MESNA thioester is displaced by the thiol of the N-terminal cysteine before an S→N acyl shift installs the peptide bond in an irreversible fashion. Alternatively, the affinity matrix is incubated with the free peptide, displacing the desired protein from the matrix and ligating in a single step.

In the specific case where there is a 1-amino-2-thiol engineered onto a C-terminal sidechain and additionally there is an N-terminal cysteine, (eg Figure 3 and Example 4) it is possible to undergo native chemical ligation on both ends of the molecule. If both 1-amino-2-thiols react with the same thioester it is possible to form a homodimer (Figure 4, Example 5). If one 1-amino-2-thiol is reacted first and another after a subsequent purification, heterodimeric structures are possible (Figure 5, Example 6).

It is also possible to incorporate more than one sidechain 1-amino-2-thiol into peptides utilising this technology to allow more than two moieties to be linked (Figure 6 and Example 7). Different lengths and compositions of linker peptide between sidechain 1-amino-2-thiols can be used to afford differing spatial and physicochemical properties.

It is also possible to introduce other reactive groups for instance an amino-oxy function on the N-terminus to allow chemically orthogonal linking to other molecules after the completion of native chemical ligation.

Any of the components brought together with this enabling technology may be selected from the group consisting of:

A pharmacophore

A ligand

A small molecule

A purification handle/immunochemical tag (eg biotin)

A fluorescent moiety

A solubilising agent

A chelating ligand

A chelating ligand plus radioimaging agent

A therapeutic protein

An antibody or fragment thereof

A peptide

An enzyme

This list is not exhaustive. Other moieties may be linked in the fashion to be described and will be apparent to those skilled in the art.

Drawings

Figure 1: Native Chemical Ligation schematic.

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Figure 2: Synthesis of a monomer to allow single-step incorporation of a protected sidechain 1-amino-2-thiol into a peptide by solid phase synthesis.

Figure 3: Structure of dimerisation reagent APT3068.

Figure 4: Preparation of a homodimer using the technology.

Figure 5: Preparation of a heterodimer using the technology.

Figure 6: Structure of trimerisation reagent APT3288.

Figure 7: IMPACT system of preparing polypeptides

Figure 8: Scheme 1 for preparing protected pseudo amino acids.

Examples

Example 1: Synthesis of disulfide exchange MSWP1 (SEQ ID NO: 1)

The peptide:
Myristoyl-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-Cys-(S-2-thiopyridyl)-NH$_2$ was synthesised according to the method described in WO 98/02454.

Example 2: Synthesis of protected lysine sidechain 1-amino-2-thiol amino acid monomer

N-α-Fmoc-N-ε-Boc-L-Lysine (5.14 g) was dissolved in 50/50

DCM/TFA (~20 mL) and stirred for 30 minutes. The solvent was

evaporated under reduced pressure and the residual yellow oil

was dissolved in water and freeze-dried to remove remaining

TFA and furnish N-α-Fmoc-L-Lysine.

A solution of Boc-Cys(Trt)-OH (4.66 g) and N-

hydroxysuccinimide (1.15 g) in 1,4-dioxane (20 mL) and DCC

(dcyclohexylcarbodiimide) (2.06 g) were stirred overnight.

The urea derivative, which precipitated, was filtered off and

washed with DCM. Solvent was evaporated and the resulting oil

dissolved in ethyl acetate, then washed with sat. sodium

carbonate solution then water. After drying over anhydrous

sodium sulphate, the solvent was removed under reduced

pressure to yield Boc-Cys(Trt)-N-hydroxy succinimide ester.

N-α-Fmoc-L-Lysine above was dissolved in DCM (20 mL) and the

Boc-Cys(Trt)-N-hydroxysuccinimide ester above dissolved in DMF

(30 mL) and DCM (10 mL). The solutions were mixed and DIPEA

added until pH 7. The mixture was stirred overnight at

ambient temperature. After this time, half the solvent was

removed under reduced pressure and ethyl acetate (200 mL)

added. The organic mixture was washed with water (100 mL),

sodium bicarbonate (200 mL), and water (100 mL). The organic

layer was dried over anhydrous sodium sulphate and evaporated

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to yield a cream coloured foam. The product was purified using

silica gel chromatography using 5:1 ethyl acetate/petroleum

ether 40-60 to afford the product as a pale cream foam.

Example 3: Synthesis of Native Chemical Ligation peptide

MSWP2278 (SEQ ID NO: 2)

The peptide:

Myristoyl-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Asp-Asp-Lys-Lys-

Pro-Gly-Asp-(ε-amino-cysteinyl carboxamide)-Lys-NH₂ was

prepared by solid phase synthesis using Boc synthesis on MBHA

resin (Nova). Coupling reactions were carried out using

appropriately protected Boc amino acid monomers (Nova)

activated with TBTU aided with HOBT (Alexis Bio. Co.) with

ninhydrin monitoring after each extension. The first amino

acid was installed as α-amino-Boc, ε-amino-Fmoc lysine, and

·the Fmoc protection then removed with 20% piperidine. α-

Amino-Fmoc, S-methoxybenzyl cysteine was then coupled to the

ε-amino group before the remainder of the synthesis was

carried out using appropriately protected Boc monomers.

Cleavage from the resin and cleavage of the sidechain

protecting groups was accomplished with high HF conditions

using p-cresol and p-thiocresol as scavengers at 0°C over 1 h.

The crude peptide was desalted via gel filtration (Sephadex

G10, 0.1% TFA in water) before purification by preparative C18

Vydac) high performance liquid chromatography (HPLC) using

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0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid/90%

acetonitrile as gradient components. After lyophilisation,

the peptide was a white amorphous powder, soluble to at least

10 mg/mL in both dimethylsulfoxide and water. Fast atom

bombardment spectrometry gave a main peak at 2101 amu

corresponding to the molecular ion of the peptide.

Example 4: Synthesis of dimerisation reagent (APT3068) (Figure 3)

Standard Fmoc solid phase synthesis was utilised on Rink amide

resin. Monomer was coupled first followed by Fmoc-9-

aminononanoic acid followed by Boc-Cys(Trt)-OH. After

cleavage from the resin and removal of all protecting groups

using TFA/EDT/TIPS (95/5/5) the crude linker was ether

precipitated and purified by preparative C18-reverse phase

HPLC. ESMS 507.2 (M+H).

Example 5: Formation of a homodimer using native chemical ligation

A solution of APT3068 in water (37.5 mM, 21.3 uL) was added to

a solution of TCEP (tris-2-carboxyethyl phosphine) (1 equiv.)

in HEPES buffer (pH 8.5, 40 mM, 265 uL). A solution of

APT2501 (10 mg/mL, 140 uL) was then added. The reaction

mixture was left at ambient temperature overnight and then

analysed by HPLC. The doubly ligated product eluted slightly

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later than APT2501 and was found to be a 2/1 ligation product

by MALDI MS - 4378. This product was purified by preparative

HPLC and lyophilised.

Example 6: Formation of a heterodimer using native chemical ligation

A solution of APT3068 in water (37.5 mM, 21.3 uL) was added to

a solution of TCEP (tris-2-carboxyethyl phosphine) (1 equiv.)

in HEPES buffer (pH 8.5, 40 mM, 265 uL). A solution of

APT2501 (1 mg/mL, 140 uL) was then added. The reaction

mixture was left at ambient temperature overnight and then

analysed by HPLC. The ligated product eluted slightly later

than APT2501 and was found to be a 1/1 ligation product by

ESMS - 2444 (M+H). This product was purified by preparative

HPLC and lyophilised. This product was a mixture of two

products with single ligation to one or other of the 1-amino-

2-thiols. These products are functionally equivalent and have

identical molecular weight. Figure 5 shows only one of these

two products for the sake of brevity.

EGFP MESNA thioester lyophilisate was dissolved in HEPES

buffer (pH 8.5 40 mM, 250 uL) and added to a solution of TCEP

(tris-2-carboxyethyl phosphine) (1 equiv.) in HEPES buffer (pH

8.5, 40 mM, 265 uL). A solution of the singly ligated product

described above in the same buffer (25 fold molar excess) was

then added and the mixture incubated overnight at ambient

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temperature. SDS PAGE showed clear and quantitative

conversion of the protein thioester to a ligated product

approximately 2 kDa higher in molecular weight.

Example 7: Formation of a trimerisation reagent (APT3288)

Standard Fmoc solid phase synthesis was utilised on Rink amide

resin. Monomer was coupled first followed by Fmoc-6-

aminocaproic acid (twice) followed by Monomer. Two further

additions of Fmoc-6-aminocaproic acid and one of Boc-Cys(Trt)-

OH completed the synthesis. After cleavage from the resin and

removal of all protecting groups using TFA/EDT/TIPS (95/5/5)

the crude linker was ether precipitated and purified by

preparative C18-reverse phase HPLC. ESMS 1035.4 (M+H).